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Determination of Leukocyte Elastase-Inhibitor Complexes and Leukocyte Neutral Proteinase Inhibitor by Enzyme Immunoassays

Leukocyte Elastase-Inhibitor Complexes in Porcine Blood, III.

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Summary: Sensitive enzyme immunoassays for the determination of total leukocyte neutral proteinase inhibitor and polymorphonuclear elastase-leukocyte neutral proteinase inhibitor complexes are described. The usable ranges of the standard curves were from 80 ng/l to 10 µg/l. The relative intra-assay coefficients of variation of the tests were between 2 and 4%, and the inter-assay coefficients of variation between 4 and 10%. In vitro and in vivo studies were performed with septic pigs and isolated leukocytes. The results show that leukocyte neutral proteinase inhibitor can be used in pigs as a parameter for the development of a septicaemia.

Introduction

During severe inflammatory processes various blood and tissue cells including polymorphonuclear granulocytes may release cellular constituents such as lysosomal proteinases and inhibitors from the cytosol extracellularly into the circulation (1–4). Such enzymes as well as oxidizing agents are released during phagocytosis and enhance the inflammatory response by degrading connective tissue structures, membrane constituents and soluble proteins by proteolysis or oxidation (2, 4).

Porcine leukocytes have been shown to contain proteolytic[1]) as well as inhibitory activities (5–7). The main inhibitory activity directed against neutral proteinases is due to leukocyte neutral proteinase inhibitor, a potent inhibitor found in the cytosol of leukocytes (7). Besides α_1-proteinase inhibitor, which is present in plasma[2]) and interstitial fluid, leukocyte neutral proteinase inhibitor represents an additional potent intracellular neutral proteinase inhibitory capacity.

The physiological function of leukocyte neutral proteinase inhibitor is of special interest and as yet not clearly unterstood. Leukocyte neutral proteinase inhibitor may, on the one hand, act as part of an intracellular defence system to regulate undesired pro-

[1]) this journal, Vol. 23, 637–643 (1985)

[2]) this journal, Vol. 23, 821–828 (1985)

teolytic activities inside the cells by complex formation with proteinases. On the other hand, it could assist the overall inhibitory potential of the organism in blood, e. g. in inflammatory processes (8), in which leukocytes release large amounts of proteinases by degranulation or during disintegration (9); leukocyte neutral proteinase inhibitor may also serve both of these purposes.

Thus leukocyte neutral proteinase inhibitor is an additional protection against proteinases in the cytosol. This is in contrast to human leukocytes, in which only small amounts of inhibitors have been described (10).

In this communication we describe the evaluation of enzyme immunoassays which can be used to measure plasma concentrations of leukocyte neutral proteinase inhibitor and its complex with polymorphonuclear leukocyte elastase[3]), and their application in experimental septicaemia in pigs. Furthermore, we report studies dealing with the release of leukocyte neutral proteinase inhibitor from polymorphonuclear granulocytes by stimulation with different agents such as endotoxin, zymosan, etc.

Material and Methods

Porcine leukocyte elastase from porcine blood was isolated as described (11). Leukocyte neutral proteinase inhibitor was purified and characterized as reported recently (7).

Antibodies directed against porcine leukocyte elastase and leukocyte neutral proteinase inhibitor were raised in rabbits using a known protocol (12). Anti-leukocyte neutral proteinase inhibitor immunoglobulin G was isolated according to *Steinbuch & Audran* (13). Anti-leukocyte neutral proteinase inhibitor IgG peroxidase conjugate was synthesized as described (14).

Enzyme immunoassay[4]) conditions

In test samples, e. g. in porcine blood, leukocyte neutral proteinase inhibitor may occur in a free, i. e. active form, or as a polymorphonuclear leukocyte elastase-inhibitor complex, or as a neutral proteinase other than elastase-inhibitor complex. For the determination of leukocyte neutral proteinase inhibitor and its complex with polymorphonuclear leukocyte elastase two enzyme immunoassays were used, ELISA type A and ELISA type B. With ELISA type A the total amount of leukocyte neutral proteinase inhibitor (free inhibitor, complex with polymorphonuclear leukocyte elastase and with another neutral proteinase, e. g. chymase) can be determined. With ELISA type B, only free inhibitor and its complex with proteinase other than elastase can be measured. The difference between ELISA type A and ELISA type B is a preincubation step in ELISA type B, in which elastase-inhibitor complex and traces of free elastase are bound to anti-polymorphonuclear leukocyte elastase antibodies fixed to a microtitre plate surface. In all assays authentic leukocyte neutral proteinase inhibitor is used as standard. Determination of the protein concentration of leukocyte neutral proteinase inhibitor solutions was performed by amino acid analysis. Uncomplexed leukocyte neutral proteinase inhibitor can be measured as described in l. c. (7).

Buffer A: 15 mmol/l Na_2CO_3, 35 mmol/l $NaHCO_3$, 3 mmol/l NaN_3, pH 9.6

Buffer B: 1.5 mmol/l KH_2PO_4, 65 mmol/l Na_2HPO_4, 137 mmol/l NaCl, 0.5 g/l Tween 20, pH 7.4 (dilute 1 : 10 before use)

Buffer C: 0.15 mmol/l KH_2PO_4, 6.5 mmol/l Na_2HPO_4, 13.7 mmol/l NaCl, 0.05 g/l Tween 20, 20 g/l bovine serum albumin, pH 7.4

Buffer D: 0.1 mol/l citric acid, 0.1 mol/l Na_2HPO_4, pH 4.5

ELISA type A

(Determination of free inhibitor and its complexes with elastase and neutral proteinase other than elastase; fig. 1 a)

Microtitre plates were coated with anti-leukocyte neutral proteinase inhibitor IgG (2 mg/l buffer A; 0.2 ml per well) at 4 °C overnight. The plates were then washed (5 times) with buffer B. Standard samples of leukocyte neutral proteinase inhibitor and test samples were diluted with buffer C. Of these samples 0.2 ml were added to the wells and the plates were incubated at 37 °C for 3 h. After incubation, the plates were washed (5 times) with buffer B; 0.2 ml of anti-leukocyte neutral proteinase inhibitor IgG peroxidase conjugate solution was then added to each well and incubated for 3 h at 37 °C. After washing, peroxidase activity was measured as described in ELISA type B. Leukocyte neutral proteinase inhibitor was used as the standard.

ELISA type B

(Determination of free inhibitor and its complex with neutral proteinase other than elastase; fig. 1 b)

Preabsorption of polymorphonuclear leukocyte elastase and its complex with elastase: microtitre plate A was coated with anti-polymorphonuclear leukocyte elastase IgG (2.5 mg/l in buffer A; 0.2 ml per well) overnight at 4 °C. After incubation microtitre plate A was washed with buffer B (5 times). Decreasing dilutions of each test sample (e. g. porcine blood) were prepared in buffer C and 0.2 ml of these samples were added to the wells. Plate A was incubated at 4 °C for 24 h to bind polymorphonuclear elastase and its complex with neutral proteinase inhibitor. A second microtitre plate B was coated in parallel with anti-leukocyte neutral proteinase inhibitor IgG (2.0 mg/l in buffer A; 0.2 ml per well) overnight at 4 °C.

Determination of leukocyte neutral proteinase inhibitor and its complex with neutral proteinase other than elastase: after incubation, microtitre plate B was washed (5 times) with buffer B and a standard solutions of leukocyte neutral proteinase inhibitor in decreasing dilutions in buffer C was added to the microtitre plate B wells. In addition, preincubated test samples (see above under preincubation step) in microtitre plate A were gently shaken for 5 min and then transferred to the corresponding wells of microtitre plate B. Titre plate B was incubated either for 3 h at 37 °C or overnight at 4 °C. After washing plate B (5 times with buffer B), 0.2 ml of anti-leukocyte neutral proteinase inhibitor IgG peroxidase conjugate solution in buffer C was added to each well and plate B was incubated again for 3 h at 37 °C. After washing, peroxidase activity was measured by adding 0.2 ml substrate solution (0.5 ml ABTS® solution (20 g/l H_2O), 9.5 ml buffer D and 0.2 ml H_2O_2 (300 g/l) to the

[3]) Enzymes
Porcine polymorphonuclear leukocyte elastase (EC 3.4.21.37, formerly EC 3.4.21.11)

[4]) Abbreviations
ELISA enzyme-linked immunosorbent assay
ABTS: 2,2'-azinobis (3-ethylbenzthiazoline) sulphonic acid
IgG: immunoglobulin G

Fig. 1 a

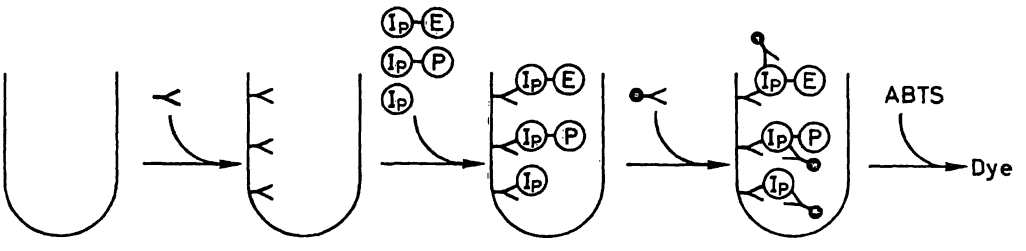

Fig. 1 b.

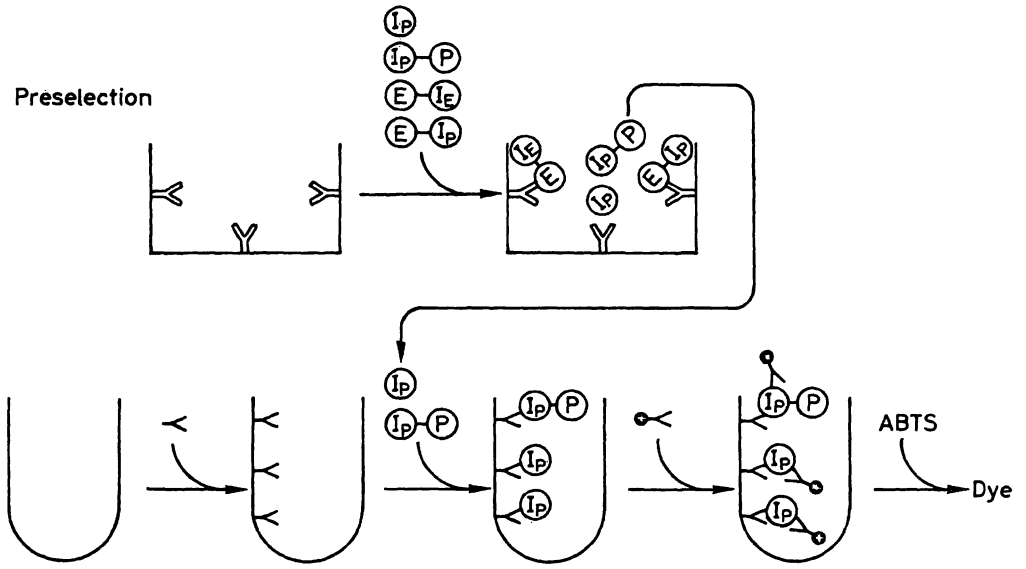

Fig. 1 a. Scheme of the enzyme immunoassay type A of leukocyte neutral proteinase inhibitor (free inhibitor and its complexes with elastase and neutral proteinase other than elastase).

Fig. 1 b. Scheme of the enzyme immunoassays type B of free inhibitor and its complexes with elastase and neutral proteinase other than elastase.
In enzyme immunoassay type B a preselection of polymorphonuclear leukocyte elastase and its complex with elastase on microtitre plates coated with anti-elastase antibodies was performed.
I_P, leukocyte neutral proteinase inhibitor; E, leukocyte elastase; P, neutral proteinase; ABTS = 2,2'-azinobis(3-ethylbenzthiazoline) sulphonic acid; ⟩—, anti-leukocyte neutral proteinase inhibitor antibodies; ⟩—●, anti-leukocyte neutral proteinase inhibitor peroxidase conjugate; ⟩═, anti-leukocyte elastase antibodies; I_E polymorphonuclear leukocyte elastase inhibitor other than leukocyte neutral proteinase inhibitor.

wells. Plate B was incubated for 30 min at 37 °C. The absorbance was read photometrically at 405 nm with a Microelisa®-reader MR 600 from Dynatech, Denkendorf, FRG. The background generally ranged from A_{405} = 0.02 to 0.08. Standard curves for leukocyte neutral proteinase inhibitor were constructed by plotting the fraction of absorbance at 405 nm, A-N/A_o-N (A: absorbance, A_o: maximal absorbance obtained with excess leukocyte neutral proteinase inhibitor, N: absorbance of the blank) against the dose of leukocyte neutral proteinase inhibitor in a semi-log mode.

Animals and cells

Septicaemia was induced in domestic pigs under pentobarbital anesthesia by infusion of 3×10^{10} live bacteria over 2 h (*E. coli*, ATCC strain 20399). Plasma samples were taken before infusion and at 2 h intervals thereafter.

Stimulation of leukocytes in whole blood was performed as follows: 6 ml whole blood and 0.12 ml stimulating reagent solution (6.96 mg zymosan, 0.06 mg endotoxin and 5×10^7 live *E. coli*) were incubated at 37 °C. Samples of 0.5 ml were taken at different time intervals and centrifuged at 12000 *g*. The supernatants were used for the determination of leukocyte neutral proteinase inhibitor, leukocyte neutral proteinase inhibitor complexes and lactate dehydrogenase. For control measurements, physiological saline was used instead of a stimulant. To stimulate leukocyte suspensions in physiological saline 6 ml of whole blood were centrifuged for 10 min at 4000 *g*. The leukocyte sediment was gently resuspended in saline, centrifuged again (in total 3 times) and suspended in 6 ml saline. The leukocyte stimulation was performed as described for whole blood.

Results and Discussion

Enzyme immunoassays for the specific determination of polymorphonuclear leukocyte elastase-leukocyte neutral proteinase inhibitor complexes and free inhibitor alone were developed. After a large series of experiments using numerous types of enzyme immunoassay conditions (combination of different antibodies for coating, etc.), the final methodology permitted an accurate determination of both elastase complexed and total leukocyte neutral proteinase inhibitor (free

inhibitor and its complexes with elastase and neutral proteinase other than elastase) in test samples. The assays were optimized with respect to

(i) enzyme immunoassay conditions (binding of antigen/antibody to microtitre plates, antibody-antigen binding, etc.) as previously described (15), and to

(ii) detection system conditions (concentration of substrates, temperatures, etc.).

Using the given incubation conditions standard curves were obtained as shown in figure 2. In both assays (ELISA type A and B) leukocyte neutral proteinase inhibitor was used as standard, because the elastase-inhibitor complex was stable for no longer than three weeks at −30 °C. Comparative studies performed with leukocyte neutral proteinase inhibitor and its elastase complex as standards showed no differences in standard curves. The lower limits of detection were about 80 ng/l in ELISA type A and 120 ng/l in ELISA type B. The intra-assay coefficients of variation (n = 10) were between 2 and 4% (ELISA type A and B); the inter-assay coefficients of variation of identical samples (n = 50) were between 4 and 9% (ELISA type A and B). The high specificity of both assays was demonstrated by adding various serine proteinases and inhibitors to the tests. No cross-reactivity was observed.

Elastase complexed and free inhibitor concentrations were determined using the newly developed enzyme immunoassays. Plasma levels of elastase complexed inhibitor were calculated by subtraction of the measured concentrations of free inhibitor and its complex with neutral proteinase other than elastase from the sum of the concentration of free inhibitor and its complexes with elastase and neutral proteinase other than elastase. The plasma concentration of neutral proteinase inhibitor and its complexes with elastase and neutral proteinases other than elastase in non-septic pigs was found to be 150 ± 30 µg/l blood, and for elastase-inhibitor complex of 16 ± 11 µg/l. In porcine leukocytes a neutral proteinase inhibitor content of 64 fg/polymorphonuclear granulocyte was measured.

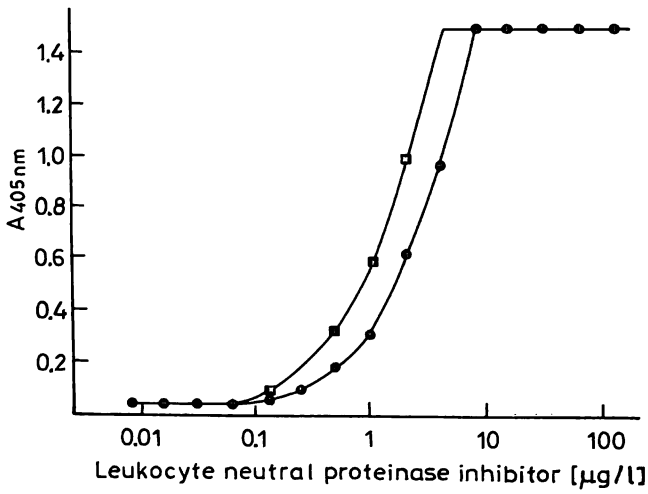

Fig. 2. Dose response curves of total leukocyte neutral proteinase inhibitor (□; free inhibitor and its complexes with elastase and neutral proteinase other than elastase) and residual leukocyte neutral proteinase inhibitor (●; free inhibitor and its complex with neutral proteinase other than elastase).

The sensitive and specific enzyme immunoassays developed for the detection of elastase complexed leukocyte neutral proteinase inhibitor and total leukocyte neutral proteinase inhibitor (free inhibitor and its complexes with elastase and neutral proteinase other than elastase) were used for model studies in pigs. Leukocyte neutral proteinase inhibitor released by desintegration of leukocytes may appear in plasma of pigs either as free inhibitor or as elastase-leukocyte neutral proteinase inhibitor complex, or complexed with other neutral proteinases, such as mast cell chymase.

During the development of septicaemia with live *E. coli*, plasma concentrations of leukocyte neutral proteinase inhibitor and its complex with elastase approx. 10 times higher than the normal physiological levels were observed in septic pigs (n = 8; fig. 3). Thus leukocyte neutral proteinase inhibitor may serve as a marker of the inflammatory response of the granulocytes in septic pigs.

In in vitro studies we stimulated both porcine leukocytes in whole blood (fig. 4a) and washed leukocytes (fig. 4b) using zymosan, live *E. coli* and endotoxin. Disintegration of leukocytes was monitored by determination of lactate dehydrogenase. As can be seen from figure 4, only zymosan induced a noticeable release of leukocyte neutral proteinase inhibitor after about 30 min. The release of leukocyte neutral proteinase inhibitor induced by *E. coli* and endotoxin is mainly due to disintegration of the cells during incubation. This is in contrast to data described for the release of elastase after stimulation of human leukocytes (16). Obviously, the inhibitor is liberated from the cells only slowly, and a potent stimulus or even disintegration of the cells may be necessary for the release reaction.

The less ready release of the inhibitor compared with that of polymorphonuclear leukocyte elastase, may be due to its localization in the cytosol of the leukocytes. Leukocyte elastase is stored in lysosomal granules of the cell, whereas leukocyte neutral proteinase inhibitor has been found only in the cytosol. Whether the release reaction is of physiological significance remains to be clarified by future studies.

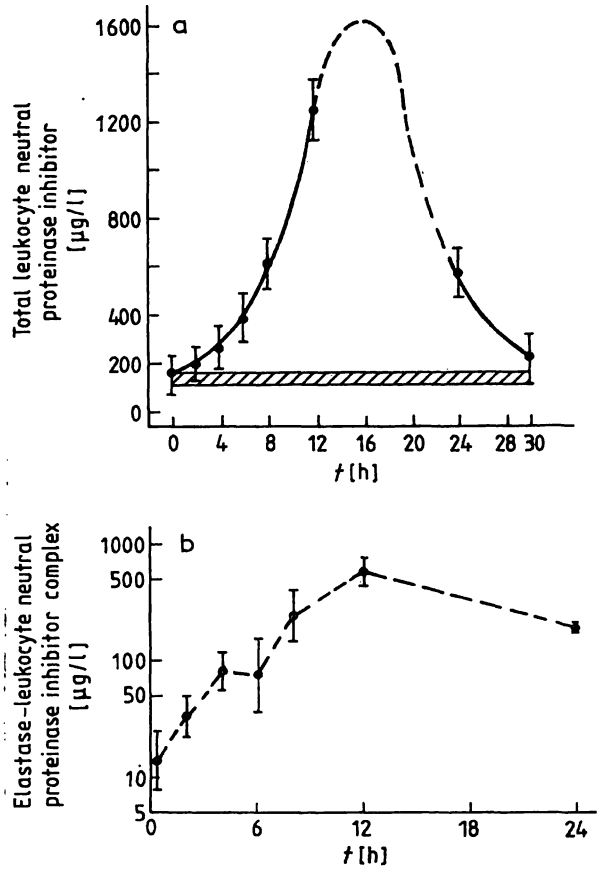

Fig. 3 a. Mean plasma concentrations of total leukocyte neutral proteinase inhibitor (free inhibitor and its complexes with elastase and neutral proteinase other than elastase) in septic pigs (n = 8).

Fig. 3 b. Mean plasma concentrations of elastase-inhibitor complex in septic pigs (n = 8).

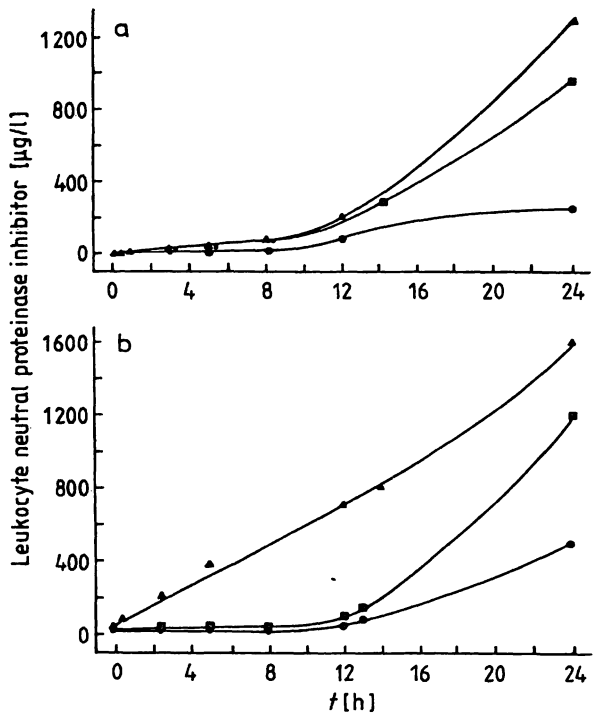

Fig. 4. In vitro stimulation of polymorphonuclear leukocytes (a, leukocytes in whole blood; b, washed leukocytes). ▲, zymosan (1 g/(l × 10^3 leukocytes); ◻, *E. coli* (5 × 10^7); ●, endotoxin (10 g/l). For details see Material and Methods.

Acknowledgement

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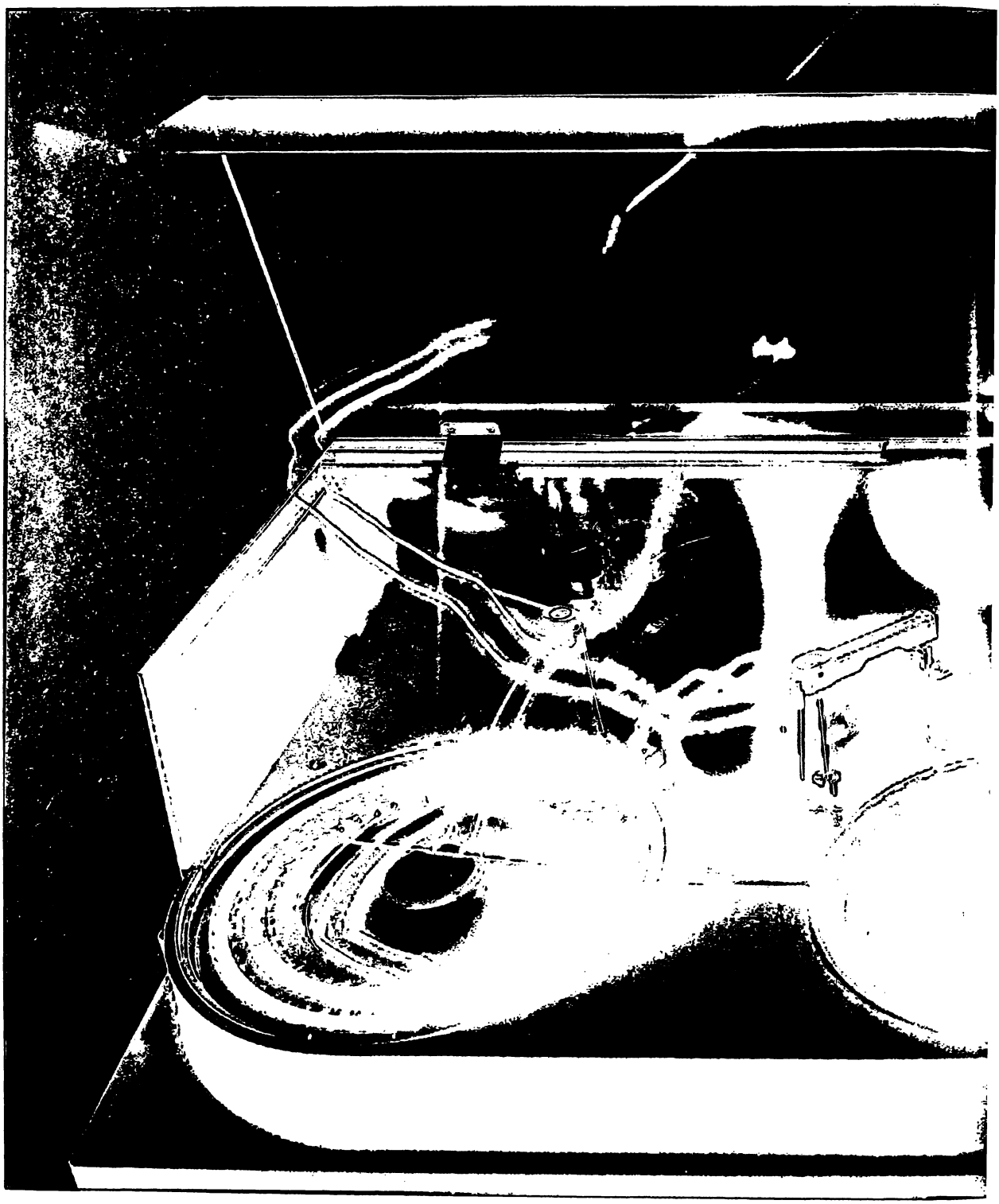

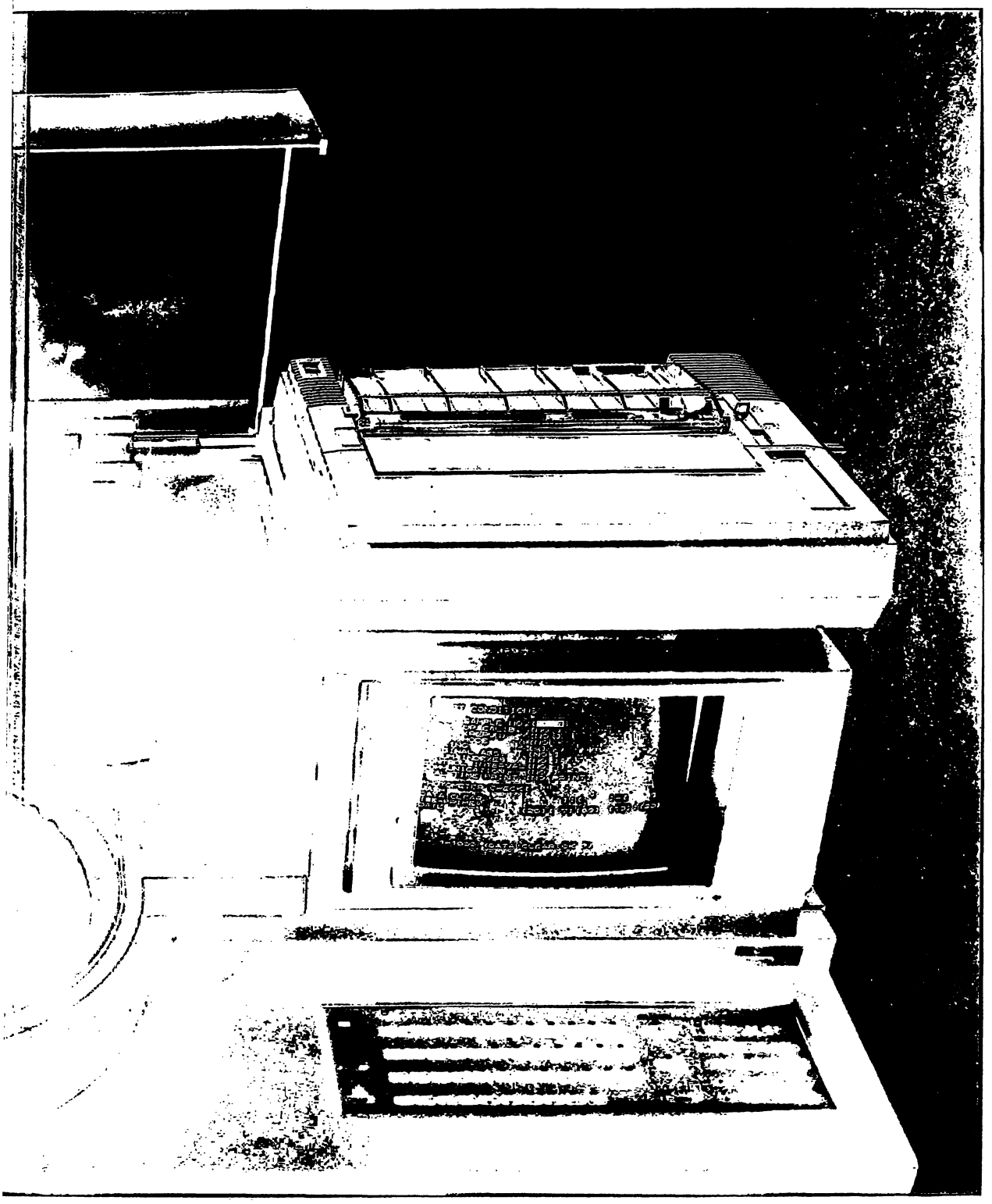

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